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PRINCIPAL INVESTIGATOR: Michael F. Press, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90089-9074

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Michael F. Press, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, California 90089-9074

E-Mail: **villalob@hsc.usc.edu**

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The HER-2/neu proto-oncogene is amplified and overexpressed in approximately 25% of breast cancers. Amplification and overexpression of HER-2/neu is correlated with poor patient prognosis, lack of responsiveness to tamoxifen treatment, responsiveness to adriamycin chemotherapy and responsiveness to Herceptin anti-HER-2/*neu* immunotherapy. In this proposal we are characterizing changes in cell adhesion related to HER-2/*neu* overexpression. We have engineered 4 human cell lines to overexpress HER-2/*neu*. Relative to parental control cell lines, HER-2/*neu* overexpressing cells lines showed altered cell adhesion on selected extracellular matrix proteins, suggesting that integrin receptors are involved in this process.

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Introduction

The HER-2/*neu* proto-oncogene is amplified and overexpressed in 25% of breast cancers. In animal models and in the human disease overexpression of this membrane receptor protein is considered to play a pathogenic role in the disease process. Clinically, overexpression is correlated with a shorter disease-free interval and a shorter overall survival of women with breast cancer. Overexpression is also correlated with lack of responsiveness to tamoxifen anti-hormone therapy and responsiveness to selected forms of chemotherapy. Immunotherapy with monoclonal antibodies to the extracellular domain of HER-2/*neu* has been associated with both partial and complete remissions of metastatic breast cancer in 10 to 20% of breast cancers with HER-2/*neu* overexpression. Although amplification and overexpression of HER-2/*neu* is clearly important in the clinical management of women with breast cancer, relatively little is known about the molecular mechanisms by which this gene alters cell physiology to produce a malignant phenotype. Most studies of HER-2/*neu* as a transforming gene have characterized tumor growth rate in culture or in xenografts, and colony formation on soft agar. None, to our knowledge, have evaluated cell adhesion to individual extracellular matrix molecules. Our primary goal in this proposal is to characterize the molecular mechanisms contributing to altered cell adhesion in HER-2/*neu* overexpressing cells.

Altered cell adhesion is an important characteristic of tumor cells because it is a necessary component of tumor cell anchorage-independent growth, ability to migrate, digest an extracellular matrix, and stimulate angiogenesis. Characterization of the molecular basis for alterations in tumor cell adhesion may provide a better understanding of the molecular mechanisms involved in more complex behaviors of tumor cells. An understanding of the mechanism(s) of altered cell adhesion may allow us to restore normal cell adhesion to HER-2/*neu* overexpressing tumor cells.

Body

Although a number of studies have shown that HER-2/*neu* oncogene overexpression causes transformation of cells, relatively little is known about the mechanism(s) by which overexpression establishes and maintains a malignant phenotype. During preliminary studies using a HER-2/*neu*-overexpressing cell line we found alterations in cell adhesion, suggesting that integrins, especially $\alpha v \beta 3$, play a role in mediating cell adhesion in tumor cells. Our working hypothesis is as follows:

HYPOTHESIS: HER-2/*neu* overexpression causes changes in cell adhesion through formation of a multimeric complex that includes the integrin receptor $\alpha v \beta 3$ and focal adhesion kinase (FAK). This causes disaggregation of integrin-mediated focal adhesions and disassociation from extracellular matrix proteins. A critical step in this process is proposed to be dephosphorylation of FAK by an as yet unidentified protein tyrosine phosphatase that interacts directly with HER-2/*neu*.

In order to address this hypothesis we have proposed accomplishing a series of tasks. Our progress with these tasks is summarized below.

Task 1. Characterize changes in cell adhesion associated with HER-2/*neu* overexpression relative to HER-2/*neu* low expression using individual extracellular matrix molecules (laminin, fibronectin, collagen, denatured collagen and vitronectin).

We previously reported that cell lines overexpressing HER-2/*neu* have alterations in adhesion to extracellular matrix proteins. Compared to parental control NIH-3T3 cells, NIH-189 cells that overexpress HER-2/*neu* showed a marked reduction in cellular adhesion to three ECM proteins: denatured collagen type I (5.8% of control), vitronectin (6.1% of control), and native collagen type I (37.3% of control). Cell adhesion to fibronectin (54.5% of control) and laminin (75% of control) was also reduced. The breast cancer cell line HBL100 was engineered to overexpress HER-2/*neu* and also demonstrated reduced adhesion to laminin, denatured collagen type I, and vitronectin. These results suggested that HER-2/*neu* overexpression interferes with integrin-mediated binding to extracellular matrix proteins.

This year we have completed the expansion of MCF-7 cells engineered to overexpress HER-2/*neu*. A single clone with a similar level of HER-2/*neu* expression as SKBR3 (a breast cancer cell line with constitutive overexpression of HER-2/*neu*) was chosen for further study. We also obtained breast cancer cell line MDA-MB-468 engineered to overexpress HER-2/*neu* (gift from?). Paired cell lines (MCF-7 and MCF-7-HER2, MDA-MB-468 and MDA-MB-468-HER2), were grown in tissue culture medium supplemented with 10% fetal bovine serum and penicillin/streptomycin for 24 h. The cells were starved of serum for 24 h to remove exogenous growth factors. Cell adhesion assays were performed in triplicate with 6 x 10^4 cells per well in 48-well cluster plates coated with individual extracellular matrix proteins (human fibronectin, human vitronectin, native collagen type I, heat-denatured collagen type I, and laminin). Poly-L-lysine coated wells were used as a control for non-integrin-mediated cell adhesion. Tumor cells were incubated in adhesion buffer (cell growth medium containing 0.5% BSA, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$) at 37°C for 20 minutes. Wells were washed three times with PBS and stained with crystal violet. Crystal violet was quantified by colorimetric analysis at 600 nm after elution with 10% acetic acid.

HER-2/*neu* overexpression reduced the adhesion of MDA-MB-468 cells to denatured collagen type I, vitronectin and laminin (30%, 57% and 50% respectively of the parental control) (Figure 1). Although the percentage reduction in cell adhesion of MDA-MB-468-HER2 cells to exracellular matrix proteins is less than that obtained with NIH-189 and HBL100-HER2 cells, the results for each cell line are qualitatively similar suggesting a common mechanism for altered cell adhesion due to overexpression of HER-2/*neu*.

Interestingly, overexpression of HER-2/*neu* in MCF-7 cells increased cell adhesion to laminin and fibronectin by 35% and 30% compared to the parental control (Figure 2). At present we are unable to explain this contradictory result; this will require a detailed analysis of the molecular changes induced by HER-2/*neu* overexpression in MCF-7 cells.

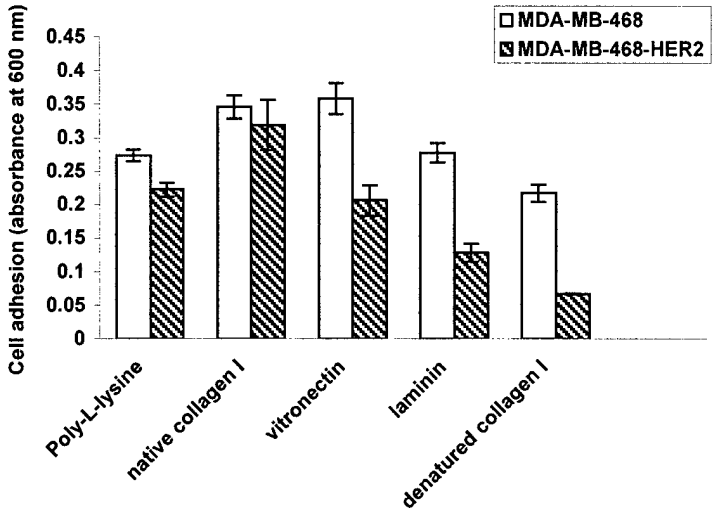

Figure 1. Adhesion of MDA-MB-468-HER2 and MDA-MB-468 parental cells to extracellular matrix proteins. Adhesion of MDA-MB-468-HER2 to denatured collagen type I, vitronectin and laminin was approximately 30%, 57% and 50% of the parental control.

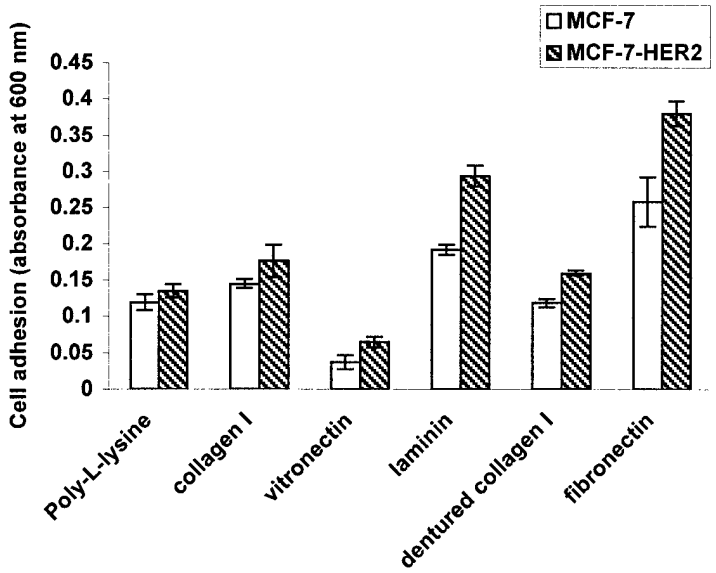

Figure 2. Adhesion of MCF-7-HER2 and MCF-7 parental cells to extracellular matrix proteins. Adhesion of MCR-7-HER2 to laminin and fibronectin was approximately 35% and 30% higher than the parental control.

Most of our research effort this year was directed at engineering two additional cell lines that overexpress HER-2/*neu*. Breast cancer cell line MDA-MB-231 and ovarian cancer cell line CaOV were stably transfected with a HER-2/*neu* expression vector by the same methods used to obtain the HBL100-HER2- and MCF-7-HER2-overexpressing cell lines. We used fluorescence-activated cell sorting (FACS) to identify and isolate transfectants with $p185^{HER\text{-}2/neu}$ overexpression. Colonies derived from single cells were dissociated and subjected to a further round of cloning. Subclones derived from these cells are currently being expanded. Cell lines that overexpress HER-2/*neu* will be identified by Western immunoblot analysis and characterized for changes in cell adhesion.

Task 2. Determine which integrins, if any, play a role in alterations of cell adhesion observed with HER-2/*neu* overexpression.

We showed previously that the $\alpha v\beta 3$ integrin receptor is probably involved in altered adhesion of HER-2/*neu* overexpressing NIH-189 and HBL100-HER2 cells. The two ECM proteins that showed the most impressive alteration in adhesion, vitronectin and denatured collagen, type I, are both associated with binding by the $\alpha v\beta 3$ integrin receptor. MCF-7-HER2 cells demonstrated increased adhesion to fibronectin and laminin. Since the $\alpha v\beta 3$ integrin receptor binds to fibronectin, we investigated whether HER-2-*neu* interacts with $\alpha v\beta 3$ in MCF-7-HER2 cells. MCF-7 cells that overexpress HER-2/*neu* (Fig. 3A), showed an association between HER-2/*neu* and integrin $\alpha v\beta 3$ in immunoprecipitation assays (Fig. 3B).
Adhesion to vitronectin and denatured collagen I is reduced in MDA-MB-468-HER2 cells. Therefore, we will analyze the interaction between HER-2/*neu* and $\alpha v\beta 3$ in these cells. Also, experiments are being done to determine the expression levels of other integrins present in both cell lines. For example, integrin $\beta 1$ is the receptor for numerous extracellular matrix proteins and may play a role in altered adhesion of MCF-7-HER2 to laminin.

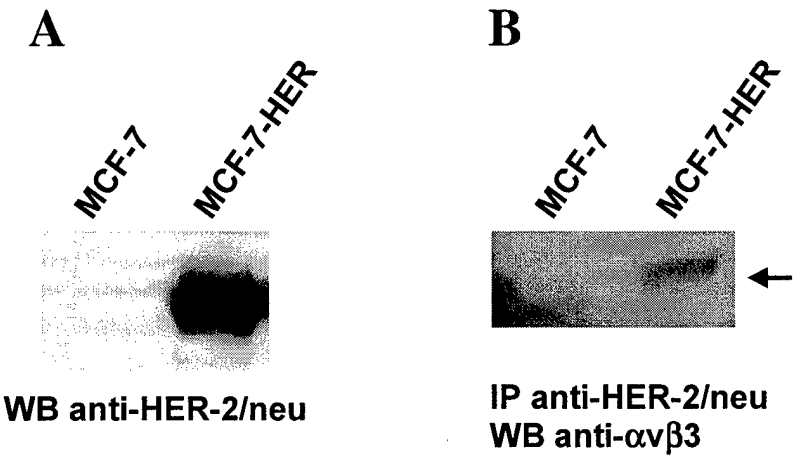

Figure 3. Association of integrin αvβ3 with HER-2/*neu* in MCF-7 and MCF-7-HER2 cells. **A**. Western immunoblot analysis demonstrates overexpression of HER-2/*neu* in MCF-7-HER2 cells compared to the parental cell line. **B**. Immunoprecipitation of HER-2/*neu* followed by Western immunoblot analysis of integrin αvβ3 demonstrates that αvβ3 associates with HER-2/*neu* in cells that overexpress of HER-2/*neu.*

Task 3. Assess the molecular mechanism responsible for alterations in cell adhesion of HER-2/*neu* overexpressing cells.

Signal transduction by integrins involves activation of intracellular protein tyrosine kinases, especially the focal adhesion-associated kinase (FAK) (1). We hypothesized that activation of FAK, as a pivotal adhesion regulatory protein, may be altered by HER-2/*neu* overexpression. In previous experiments we showed that although FAK was present in approximately equal amounts in NIH-3T3 and NIH-189 cells, only FAK from control cells demonstrated more than trace phosphorylation of tyrosine residues. This suggested that FAK was either not phosphorylated or that it was dephosphorylated after association with HER-2/*neu.* Thus, our current working hypothesis for the mechanism of action of HER-2/*neu* involves association of a protein tyrosine phosphatase (PTP) with HER-2/*neu* - FAK to remove phosphate groups from FAK. A family of such protein tyrosine phosphatases activated by tyrosine phosphorylation has been described, one of which (PTP-1D) is known to associate with HER-2/*neu* (2). Although HER-2/*neu* phosphotyrosine residues are unaltered by this association, the substrate for this PTP has not been identified (2). We investigated the role of PTP-1D in altered cell adhesion of NIH-189 cells by Western blot and immunoprecipitation analysis. Figure 4A shows that NIH-3T3 and NIH-189 cells contain equivalent amounts of PTP-1D. When PTP-1D was immunoprecipitated from cell lysates and probed with anti-phosphotyrosine antibody, we observed that PTP-1D was only phosphorylated in cells that overexpress HER-2/*neu* (Figure 4B). Also, PTP-1D was associated with HER-2/*neu* in NIH-189 cells. These results suggest that HER-2/*neu* forms a complex with and phosphorylates PTP-1D in NIH-189 cells. That PTP-1D is activated in HER-2/*neu* overexpressing cells

supports our current hypothesis. At present, we are investigating whether loss of FAK phosphorylation is due to its association with PTP-1D.

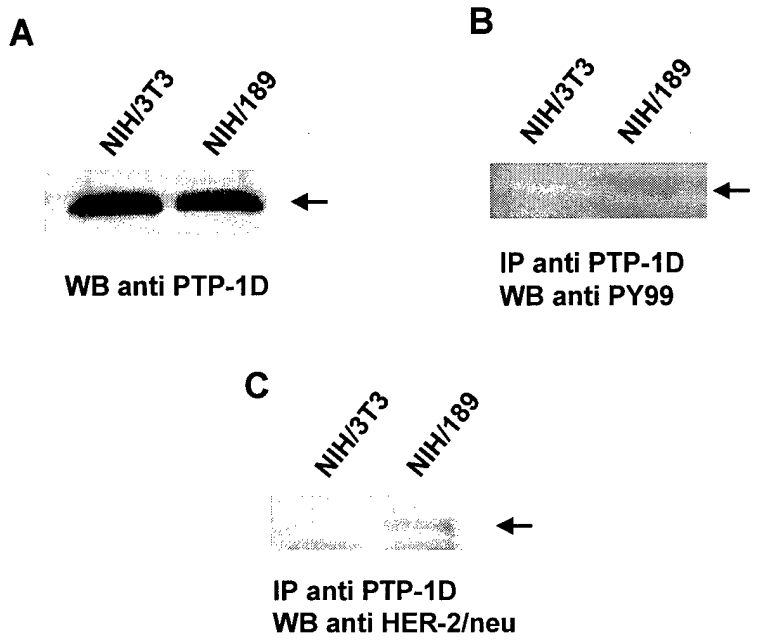

Figure 4. Phosphorylation of PTP-1D in NIH-189 cells and its association with HER-2/*neu*. **A.** Cell lysates were probed with antibody to PTP-1D. **B.** Cell lysates were immunoprecipitated with anti-PTP-1D antibody and probed with anti-phosphotyrosine (PY99). **C.** Cell lysates were immunoprecipitated with anti-PTP-1D and probed with anti-HER-2/*neu* antibody.

We have begun to investigate the effects of HER-2/*neu* on expression of FAK in MDA-MB-468-HER2 and MCF-7-HER2 cells. Previous experiments showed that there are equivalent amounts of FAK in NIH-3T3 and NIH-189 cells and that HER-2/*neu* associates with FAK in NIH-189 cells. MDA-MB-468-HER2 and MCF-7-HER2 cells have elevated levels of FAK compared to the parental controls (Figures 5 and 6). This is an interesting observation given the results of a separate study where we found increased levels of migration in HER-2/*neu* overexpressing cells. Others have shown that overexpression of FAK is correlated to an increase in cell migration (3). Like NIH-189 cells, MCF-7-HER2 contained HER-2/*neu* in association with FAK. Presently, we are investigating the activation state (phosphorylation) of FAK in MDA-MB-468-HER2 and MCF-7-HER2 cells.

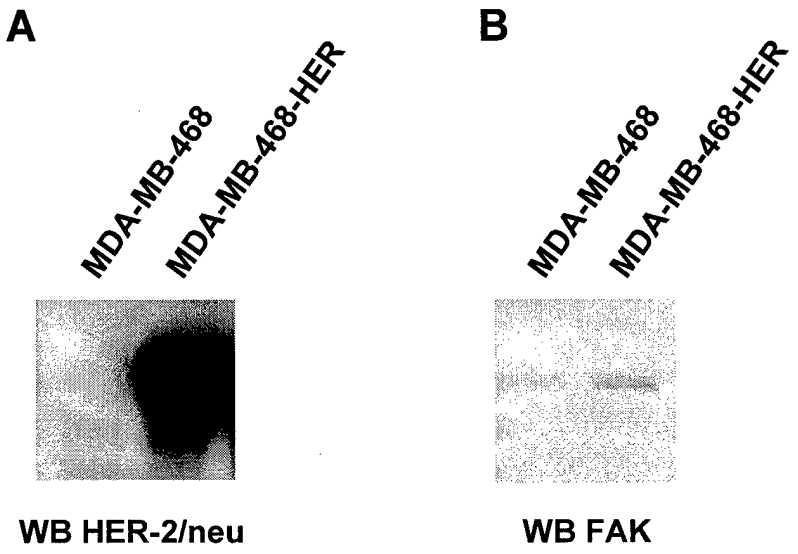

Figure 5. Expression of FAK in MDA-MB-468 and MDA-MB-468-HER2 cells. **A.** MDA-MB-468-HER2 cells overexpress the human $p185^{HER\text{-}2/neu}$ membrane receptor, whereas parental cells do not. **B.** Cells overexpressing HER-2/*neu* contain elevated levels of focal adhesion kinase (FAK).

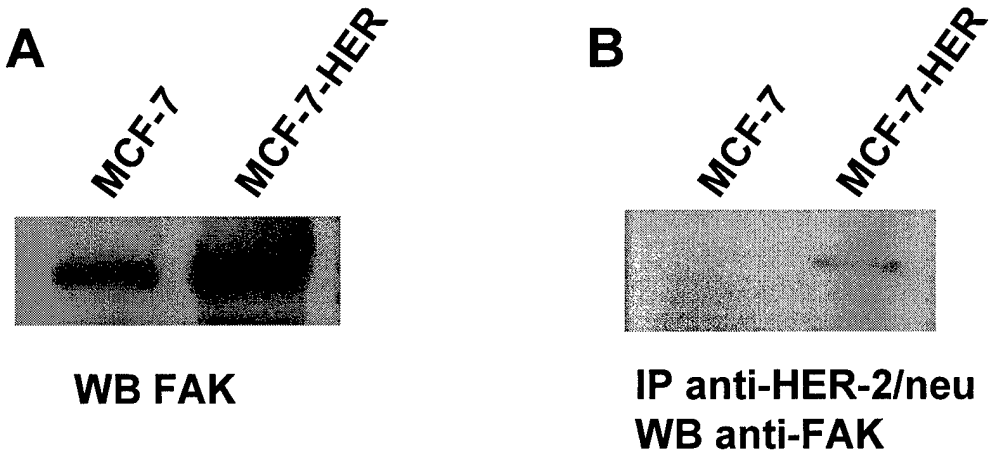

Figure 6. Expression of FAK in MCF-7 and MCF-7-HER2 cells. **A.** MCF-7-HER2 cells contain elevated levels of FAK compared to the parental control cell line. **B.** FAK is associated with HER-2/*neu* in MCF-7-HER2 cells.

Key Research Accomplishments

The research accomplishments described in the Body of this Progress Report for the last year can be summarized as follows:

1) Two human breast cell lines (MDA-MB-231 and CaOV) have been stably transfected with a HER-2/*neu* expression vector to produce engineered cell lines that differ from their parental control cell lines only with regard to the level of HER-2/*neu* expression.

2) We have shown that HER-2/*neu* overexpression is associated with altered cell adhesion to specific extracellular matrix proteins implicating integrin receptors as mediators of altered cell adhesion.

3) PTP-1D is phosphorylated and associates with HER-2/*neu* in NIH-189 cells that overexpress HER-2/*neu*.

4) FAK is elevated in MCF-7 and MDA-MB-468 cells that overexpress HER-2/*neu*.

Reportable Outcomes

Development of a stably transfected human breast cancer cell line with HER-2/*neu* overexpression (MDA-MB-468) and a stably transfected ovarian cancer cell line with HER-2/*neu* overexpression (CaOV).

Conclusions

HER-2/*neu* overexpression is associated with alterations in cell adhesion to specific extracellular matrix proteins. The changes in cell adhesion implicate integrin receptors in mediating this process. PTP-1D is phosphorylated in NIH-189 cells suggesting that this molecule may be involved in the dephosphorylation of FAK in HER-2/*neu* overexpressing cells. Changes in cell adhesion are an important part of the process by which a tumor cell migrates or becomes metastatic to other sites in the body.

References

1. Guan, J.-L. and Shalloway, D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature, 358: 690-692, 1992.

2. Vogel, W., Lammers, R., Huang, J., and Ullrich, A. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science, 259: 1611-1613, 1993

3. Cary L.A., Chang J.F., and Guan J.L. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Sci., 109:1787-94, 1996